# EXHIBIT A

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# Inhibition by Boswellic Acids of Human Leukocyte Elastase

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## ABSTRACT

Frankincense extracts and boswellic acids, biologically active pentacyclic triterpenes of frankincense, block leukotriene biosynthesis and exert potent anti-inflammatory effects. Screening for additional effects of boswellic acids on further proinflammatory pathways, we observed that acetyl-11-keto-β-boswellic acid, an established direct, nonredox and noncompetitive 5-lipoxygenase inhibitor, decreased the activity of human leukocyte elastase (HLE) *in vitro* with an $IC_{50}$ value of about 15 μM. Among the pentacyclic triterpenes tested in concentrations up to 20 μM, we also observed substantial inhibition by β-boswellic acid, amyrin and ursolic acid, but not by 18β-glycyrrhetinic acid. The data show that the dual inhibition of 5-lipoxygenase and HLE is unique to boswellic acids: other pentacyclic triterpenes with HLE inhibitory activities (*e.g.*, ursolic acid and amyrin) do not inhibit 5-lipoxygenase, and leukotriene biosynthesis inhibitors from different chemical classes (*e.g.*, NDGA, MK-886 and ZM-230,487) do not impair HLE activity. Because leukotriene formation and HLE release are increased simultaneously by neutrophil stimulation in a variety of inflammation- and hypersensitivity-based human diseases, the reported blockade of two proinflammatory enzymes by boswellic acids might be the rationale for the putative antiphlogistic activity of acetyl-11-keto-β-boswellic acid and derivatives.

Frankincense is a gum resin secreted by trees of the genus *Boswellia* of Burseraceae. From the very beginning of human civilization, it has been used for therapeutic purposes (Martinetz *et al.*, 1988). In Europe, it was a component of the pharmacopoeia until the beginning of this century, and then, with the onset of the era of synthetic drugs, it fell into oblivion. Frankincense is still used in the region from North Africa to China as a remedy, especially in the traditional Ayurvedic medicine of India. In the eighties, it was reported that an ethanolic extract of *Boswellia* gum exerted anti-inflammatory and antiarthritic activities in animals (Singh and Atal, 1986; Reddy *et al.*, 1987). In an effort to find novel biologically active principles from plant origin, we observed that frankincense extracts inhibited leukotriene biosynthesis *in vitro* (Ammon *et al.*, 1991). As active principles, we identified boswellic acids that belong to ursane-type pentacyclic triterpene saponines, and we demonstrated that boswellic acids selectively blocked leukotriene biosynthesis (Safayhi *et al.*, 1992). The boswellic acid derivative AKBA inhibited 5-LO, the key enzyme of leukotriene biosynthesis, by an enzyme-directed, nonredox and noncompetitive mechanism *via* binding to a pentacyclic triterpene-selective effector site (Safayhi *et al.*, 1995; Sailer *et al.*, 1996a).

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However, in 1991 we observed that boswellic acids also prevent endotoxin-/galactosamine-induced hepatitis in mice (Safayhi *et al.*, 1991). This observation was intriguing, because it had been reported that protection against endotoxic shock could be achieved only by less selective lipoxygenase blockers, not by site-specific leukotriene biosynthesis inhibitors (Schade *et al.*, 1991, Schade *et al.*, 1992), and that 5-LO-deficient transgenic mice showed no difference in their reaction to endotoxin shock (Chen *et al.*, 1994). In 1991, it was reported that the pentacyclic triterpene ursolic acid inhibited HLE (EC 3.4.21.37) (Ying *et al.*, 1991). HLE is a serine protease produced and released by PMNL, and because of its aggressive destructiveness, some investigators have suggested that HLE may play a role in several diseases, such as pulmonary emphysema, cystic fibrosis, chronic bronchitis, acute respiratory distress syndrome, glomerulonephritis and rheumatic arthritis (for review see Bernstein *et al.*, 1994). In 1995, it was demonstrated that granulocyte-mediated hepatotoxicity after endotoxin stimulation depends on elastase release (Sauer *et al.*, 1995).

The aim of this study was to determine whether the established pentacyclic triterpene-type 5-LO inhibitor AKBA also affects the activity of HLE. Here, we report that many pentacyclic triterpenes, including the boswellic acids, block HLE activity *in vitro* but that the combined inhibition of two

**ABBREVIATIONS:** AKBA, acetyl-11-keto-β-boswellic acid; DMSO, dimethylsulfoxide; HLE, human leukocyte elastase; 5-LO, 5-lipoxygenase; $LTB_4$, leukotriene $B_4$; MK-886 (formerly designated L-663,536), 3-[1-(4-chlorobenzyl)-3-tert-butyl-thio-5-isopropylindol-2-yl-]-2,2-dimethylpropanoic acid; NDGA, nordihydroguaiaretic acid; PBS, Dulbecco's phosphate-buffered saline; PMNL, polymorphonuclear leukocytes; ZM-230,487 (formerly designated ICI-230,487; the N-ethyl-analog of ICI-D2138), 6-[[3-fluoro-5-(4-methoxy-3,4,5,6-tetrahydro-2H-pyran-4-yl)phenoxy]methyl]-1-ethylquinol-2-one.

pathophysiologically important enzyme activities (those of HLE and 5-LO) in an independent manner is unique to pentacyclic triterpenes from the boswellic acid series.

## Materials and Methods

**Chemicals.** Ursolic acid, 18β-glycyrrhetinic acid, amyrin (a mixture of isomeric α- and β-forms) was purchased from Roth (Karlsruhe, FRG, both Rotichrom GC grade). AKBA and β-boswellic acid were purified and characterized by spectroscopy (infrared, $^1$H-NMR and mass) (see fig. 1 for structures), by thin-layer chromatography, by elemental analyses and by their melting points, as described in detail elsewhere (Safayhi *et al.*, 1992; Sailer *et al.*, 1996b). NDGA, testosterone, cortisol, arachidonic acid (Na-salt), Suc-Ala-Ala-Pro-Phe-p-nitroanilide, MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide and $\alpha_1$-antitrypsin were obtained from Sigma (Deisenhofen, FRG). HLE was obtained from Calbiochem (Bad Soden, FRG), and chymotrypsin from Boehringer (Mannheim, FRG). MK-886 and ZM-230,487 (formerly ICI-230,487) were kind gifts from Dr. A.W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Kirkland, Canada) and from Dr. G.C. Crawley (ICI & Zeneca Pharmaceuticals, Macclesfield, Cheshire, England), respectively.

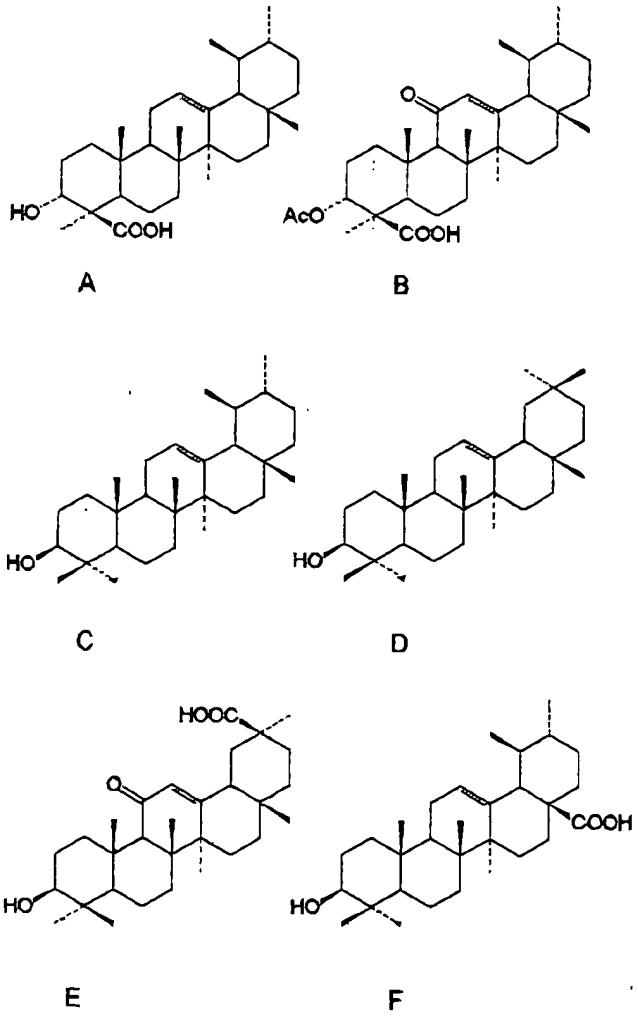


Fig. 1. Chemical structures of the pentacyclic triterpenes used in the present study A) β-boswellic acid; B) AKBA; C) α-amyrin; D) β-amyrin; E) 18β-glycyrrhetinic acid; F) ursolic acid).

**Measurement of HLE activity.** The hydrolytic activity of HLE was measured using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as substrate in PBS containing 10% DMSO (v/v) at 25°C (Bieth *et al.*, 1974). Enzyme (20 nM) was preincubated for 5 min in the presence of test compounds or vehicle (DMSO). The final concentration of DMSO was 10.25% throughout. The reaction was started by the addition of substrate. The formation of p-nitroanilide (pNA) was monitored by detection at 405 nm for 5 min. Using a substrate concentration range from 10 μM to 4 mM we calculated a $K_m$ value of about 148 to 198 μM and a $V_{max}$ value of about 52 to 57 nanomoles per second for the commercial enzyme preparation, the variation depending on the linearization procedures used.

**Measurement of chymotrypsin activity.** The hydrolytic activity of chymotrypsin was measured using Suc-Ala-Ala-Pro-Phe-pNA as substrate in a Tris buffer containing 10 mM $CaCl_2$ at 25°C (Del-Mar *et al.*, 1979). Enzyme (40 nM) was preincubated for 5 min in the presence of test compounds or vehicle (DMSO). The reaction was started by the addition of substrate in DMSO. All incubations, including controls, were carried out in the presence of 10.25% DMSO. The formation of pNA was monitored by detection at 410 nm for 5 min.

**Data.** Product formation was calculated by comparison with a standard curve for pNA. Data on observations ($n$ = number of experiments) are shown as means ± S.D. Enzyme kinetic data were analyzed by constructing Lineweaver-Burk and Eadie-Hoffstee plots (Bisswanger, 1979). The $IC_{50}$ values were calculated by using GraphPad Prism software, version 2.0, for one-site competition (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed using Student's $t$ test for unpaired data.

## Results

The pentacyclic triterpene AKBA, a direct, nonredox and noncompetitive 5-lipoxygenase inhibitor, blocked the hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-pNA by HLE in a concentration-dependent manner, as shown in figure 2. The $IC_{50}$ value for AKBA was 13.8 ± 2.0 μM ($n$ = 5). The pentacyclic triterpene ursolic acid, which possesses no 5-LO inhibitory properties, blocked the activity of HLE with $IC_{50}$ values of 0.9 ± 0.6 μM (at 50 μM substrate, $n$ = 3) to 2.4 ± 0.2 μM (at 500 μM substrate, $n$ = 3). Among the pentacyclic triterpenes, a substantial elastase inhibition was also observed by β-bo-

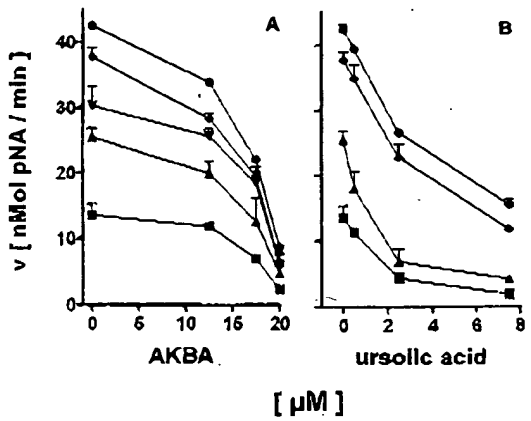


Fig. 2. Inhibition of HLE activity by AKBA (panel A) and ursolic acid (panel B). Substrate (MeO-Suc-Ala-Ala-Pro-Val-pNA) concentrations were 50 (■), 100 (▲), 150 (▼), 300 (◆) and 500 μM (●). The assays were carried out in PBS/10.25% DMSO, pH 7.2, at 25°C. The enzyme concentration was 20 nM. Data are shown as absolute values of pNA release, in nanomoles per minute, as means ± S.D. of three experiments.

swellic acid and amyrin, but not by 18β-glycyrrhetic acid in concentrations up to 20 μM (table 1). The HLE activity was also not decreased by various other noncyclic or cyclic lipophilic compounds (*e.g.*, arachidonic acid, cortisol and testosterone) in comparable concentrations.

Again in contrast to the inhibitory effect of the direct, nonredox and noncompetitive 5-LO inhibitor AKBA on HLE, other leukotriene biosynthesis inhibitors from different chemical classes exerted no HLE inhibitory activity. As shown in table 1, no substantial inhibition of HLE was observed by the redox-type 5-LO inhibitor NDGA, by the so-called translocation inhibitor MK-886 or by the nonredox-type-competitive 5-LO inhibitor ZM-230,487.

As illustrated in figures 3 and 4 by secondary Lineweaver-Burk and Eadie-Hofstee plots, data analyses indicate different mechanisms for the inhibitory actions of the pentacyclic triterpenes AKBA and ursolic acid. The mode of inhibition was noncompetitive with AKBA but competitive with ursolic acid.

In order to determine whether AKBA also impairs nonselectively the activities of other serine proteases, we evaluated its effect on chymotrypsin activity. As shown in table 2, no prominent inhibition by AKBA of chymotrypsin was observed in concentrations up to 100 μM, whereas ursolic acid decreased the chymotrypsin activity by about 70% at a high concentration of 100 μM.

## Discussion

The boswellic acid derivatives AKBA and β-boswellic acid, as well as amyrin, inhibited the hydrolysis of a synthetic substrate by purified HLE *in vitro*, as was previously reported for other pentacyclic triterpenes (*i.e.*, ursolic acid, oleanolic acid, uvaol and erythrodiol (Ying *et al.*, 1991). Although the *in vitro* test system that we used contains substantial amounts of organic solvent and, therefore, would have permitted the addition of test compounds in greater quantities for screening purposes, we limited the final concentrations to 20 μM because higher plasma levels are not likely with the lipophilic pentacyclic triterpenes. With 20 μM

TABLE 1

**HLE activity in the presence of various cyclic and noncyclic hydrophobic compounds and leukotriene biosynthesis inhibitors**

The assay was performed using MeO-Suc-Ala-Ala-Pro-Val-pNA as substrate in PBS, pH 7.2, containing 10.25% DMSO at 25°C. The HLE concentration was 20 nM, and the substrate concentration was 100 μM. Test compounds were assayed at a final concentration of 20 μM throughout. Data are shown as absolute values of pNA release in nanomoles per minute (mean ± S.D.; $^{***}P < .001$ vs. DMSO controls) in three experiments or percent of HLE activity in controls.

| Addition (20 μM) | HLE Activity | |
|---|---|---|
| | (nmol/min) | (% of control) |
| None | 25.0 ± 2.0 | 100 |
| AKBA | 3.8 ± 1.5*** | 15 |
| β-Boswellic acid | 9.2 ± 0.8*** | 36 |
| Ursolic acid | 3.3 ± 0.4*** | 13 |
| Amyrin | 8.1 ± 0.5*** | 32 |
| 18β-Glycyrrhetinic acid | 23.8 ± 2.3 | 93 |
| Cortisol | 23.1 ± 2.9 | 91 |
| Testosterone | 23.8 ± 1.8 | 93 |
| Arachidonic acid | 22.1 ± 1.5 | 87 |
| NDGA | 21.9 ± 2.7 | 86 |
| MK-886 | 21.1 ± 2.4 | 83 |
| ZM-230,487 | 22.5 ± 2.6 | 88 |

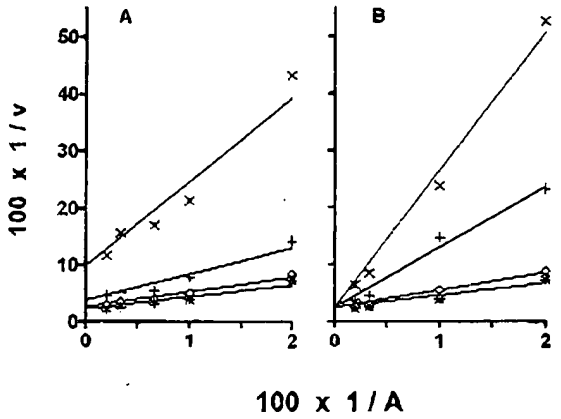


Fig. 3. Lineweaver-Burk plots of the HLE inhibition by AKBA (panel A) and ursolic acid (panel B) with MeO-Suc-Ala-Ala-Pro-Val-pNA as substrate. Velocity (*v*) is expressed in nanomoles pNA per minute, and the substrate concentration (*A*) in micromoles per liter. Substrate concentrations were 50, 100, 150, 300 and 500 μM in panel A and 50, 100, 300 and 500 μM in panel B. Inhibitor concentrations were 0 (*), 12.5 (◇), 17.5 (+) and 20 (×) μM AKBA in panel A, and 0 (*), 1 (◇), 2.5 (+) and 7.5 (×) μM ursolic acid in panel B.

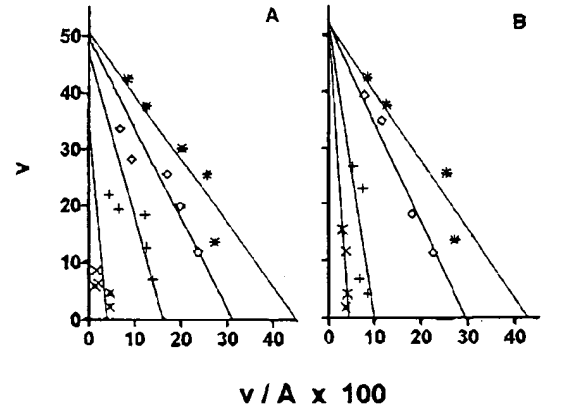


Fig. 4. Eadie-Hofstee plots of the HLE inhibition by AKBA (panel A) and ursolic acid (panel B) with MeO-Suc-Ala-Ala-Pro-Val-pNA as substrate. Velocity (*v*) is expressed in nanomoles pNA per minute, and the substrate concentration (*A*) in micromoles per liter. Substrate concentrations were 50, 100, 150, 300 and 500 μM in (panel A) and 50, 100, 300 and 500 μM in (panel B). Inhibitor concentrations were 0 (*), 12.5 (◇), 17.5 (+) and 20 (×) μM AKBA in (A); 0 (*), 1 (◇), 2.5 (+) and 7.5 (×) μM ursolic acid in (B).

in each case, we observed *in vitro* no substantial HLE inhibition by 18β-glycyrrhetinic acid, cortisol, testosterone or arachidonic acid.

We previously reported that many pentacyclic triterpenes also bind to 5-LO, the key enzyme of leukotriene biosynthesis (Safayhi *et al.*, 1995). The presence of an 11-keto-group and a hydrophilic function on ring A of the pentacyclic ring system are crucial for potent inhibition of 5-LO, and ursolic acid and amyrin turned out to be noninhibitory (Sailer *et al.*, 1996a). Thus the structure requirements for the 5-LO inhibitory activity of pentacyclic triterpenes are more rigid than those for HLE inhibitory activity. Our data are in line with the hypothesis that pentacyclic triterpenes interact with the extended substrate binding domain in the HLE that can accommodate a variety of hydrophobic ligands (Ashe and Zimmerman, 1977; Cook and Ternai, 1988; Ying *et al.*, 1991). With a

TABLE 2

**Chymotrypsin activity in the presence AKBA, ursolic acid and $\alpha_1$-antitrypsin with Suc-Ala-Ala-Pro-Phe-pNA as substrate**

The assay was performed in PBS containing 10.25% DMSO, pH 7.2, at 25°C. Final concentrations were 40 nM for chymotrypsin, 100 μM for substrate and 3.8 μM for $\alpha_1$-antitrypsin ($n$ = 3; *P < .05; **P < .01 and ***P < .001).

| Addition | Chymotrypsin Activity | |
|---|---|---|
| | (nmol/min) | (% of controls) |
| None | 55.4 ± 2.7 | 100 |
| Ursolic acid | | |
| 25 μM | 44.9 ± 0.9** | 81 |
| 50 μM | 31.4 ± 4.4*** | 57 |
| 100 μM | 16.6 ± 4.1*** | 30 |
| AKBA | | |
| 50 μM | 52.4 ± 2.2 | 95 |
| 100 μM | 49.9 ± 1.1* | 90 |
| $\alpha_1$-antitrypsin | 0 | 0 |

pentapeptide substrate, we observed competitive-type HLE inhibition by ursolic acid, but a noncompetitive mode of inhibition by AKBA (figs. 3 and 4). The reason for this difference is not obvious, but it is a general property of HLE inhibition. For example, oleic acid derivatives have been described as both competitive and noncompetitive inhibitors of HLE (Tyagi and Simon, 1990; Ashe and Zimmerman, 1977; Hornebeck *et al.*, 1995), and, depending on substrate length, different mechanisms have also been reported for ursolic acid (Ying *et al.*, 1991).

In summary, boswellic acids with 5-LO inhibitory activity block HLE activity. HLE inhibition is established for many lipophilic compounds, but a dual HLE and 5-LO inhibitory property is unique to pentacyclic triterpenes from the boswellic acid series. Because leukotriene levels and HLE release are increased in parallel in many inflammatory diseases and hypersensitivity-based reactions (Mayatepek and Hoffmann, 1995; Bernstein *et al.*, 1994), boswellic acid derivatives such as AKBA might provide a tool to help us cope better with such pathophysiological processes. In line with this hypothesis, boswellic acid containing crude extracts of the *Boswellia* resin have been recently reported to inhibit the increased urinary excretion of leukotriene $E_4$ in astrocytoma patients *in vivo* and to block leukotriene biosynthesis *ex vivo* (Heldt *et al.*, 1996).

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